# Natural photosensitizers in constructed unit process wetlands: Photochemical characterization and inactivation of pathogen indicator organisms

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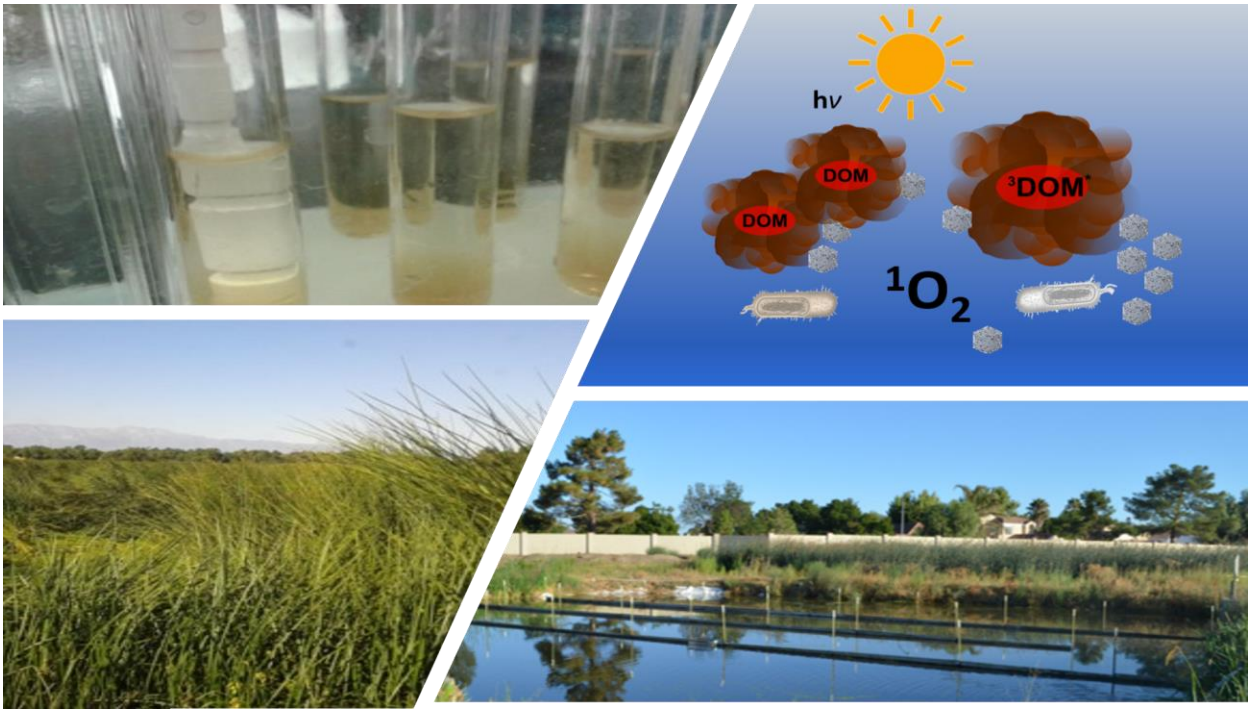



**Graphical Abstract**

**Abstract**

Dissolved organic matter (DOM) is a natural photosensitizer that contributes to the inactivation of microbial pathogens. In constructed treatment wetlands with open water areas DOM can promote sunlight disinfection of wastewater effluent, but a better understanding of DOM spectroscopic and photochemical properties and how they are impacted by different unit process wetlands is needed to inform design. The goals of this study were: (1) to investigate whether DOM isolates realistically represent the photochemistry of the source DOM in its original water; and (2) to observe how changes of DOM along a treatment wetland affect its photochemistry, including pathogen inactivation. A pilot scale unit process wetland was studied that consisted of three different cells (open water, cattail, and bulrush) fed by secondary wastewater effluent. DOM was isolated using solid-phase extraction (SPE), photochemically characterized, and compared to the original water samples and standard DOMs. Both excited triplet state and singlet oxygen quantum yields decreased in the vegetated wetlands indicating that predominantly light-absorbing or reactive species quenching DOM was released by the wetland vegetation. For MS2 coliphage, a virus indicator, the most efficient photosensitizer was the wastewater DOM isolated from the influent of the wetland, while for the bacterial indicator *Enterococcus faecalis,* inactivation results were comparable across wetland isolates. SPE resulted in isolation of 47% to 59% of whole water DOM and enriched for colored DOM. Singlet oxygen precursors were efficiently isolated, while some excited triplet state precursors remained in the extraction discharge. DOM processing indicators such as $SUVA_{254}$, $SUVA_{280}$ and spectral slopes including $E_2/E_3$ ratios were reflected in the isolates. Photoinactivation of MS2 was significantly lower in both the reconstituted water samples and isolates compared to the original water sample, possibly due to disturbance of the trans-molecular integrity of DOM molecules by SPE that affects

distance between MS2 and DOM sites with locally higher singlet oxygen production. For *E. faecalis* results were similar in original water samples and isolates. Higher sorption of DOM to *E. faecalis* was roughly correlated with higher photoinactivation rates. To enhance sunlight disinfection in unit process wetlands, there is no advantage to placing open water cells after vegetated cells, as passage through the vegetated cells led to increased light absorption and lower singlet oxygen and triplet-state quantum yields and steady state concentrations.

## 1. Introduction

Constructed wetlands can be a low-energy, low-cost method of wastewater treatment.[1, 2] However, adoption has been limited by the difficulty in reliably predicting their performance and challenges treating some contaminant classes, including pathogens and trace organic chemicals.[3] One way to overcome these limitations is to build wetland systems consisting of distinct units, with each unit having a distinctive design, customized to treat a particular suite of contaminants.[4, 5] Among such unit processes, shallow sunlit open water wetland cells can be used to remove trace organic chemical contaminants that are susceptible to photochemical transformation and to inactivate pathogenic microbial contaminants such as waterborne bacteria and viruses.[6-12]

For pathogens, there are three sunlight inactivation mechanisms: direct inactivation, indirect endogenous and indirect exogenous inactivation. Direct inactivation occurs when chromophores (e.g., DNA or protein) that are part of the microorganism are damaged by the absorption of UVB-light (280 – 320 nm). Indirect endogenous inactivation occurs when chromophores that are part of the microorganism sensitize the formation of photo-produced reactive intermediates (PPRI) that cause damage whereas exogenous inactivation is caused by PPRI that are generated by sensitizers outside the organism.[13]

The exogenous mechanism has the potential to contribute significantly in surface waters containing chromophoric dissolved organic matter (CDOM), in which penetration of shorter UV wavelengths is limited.[14-16] Under some conditions, exogenous sensitizers have been shown to enhance the sunlight inactivation of human viruses (poliovirus type 3,[17] adenovirus type 2,[15 17] human Wa and porcine OSU rotaviruses[18, 19]); bacteriophages (MS2,[17, 20, 21], PRD1[17] and phiX174[22]); health-related bacteria, including gram-positive species (*E. faecalis*,[14, 15, 23] *Staphylococcus aureus*,[14, 15] *Streptococcus bovid* [14, 15]), and gram-negative species (*Escherichia coli*,[14, 15, 23] and *Campylobacter jejune*[14, 15]).

Although DOM reduces the penetration of light into surface waters, which reduces the rate of direct photochemical processes,[24] it is also the main sensitizer involved in the formation of PPRI in surface waters.[25, 26] The PPRI include excited triplet states of dissolved organic matter ($^3DOM^*$), singlet oxygen ($^1O_2$), hydroxyl radical $^\bullet OH$) and the carbonate radical ($^\bullet CO_3^-$).[27, 28] Besides being the source of the reactive species, DOM also serves as a sink for reactive species, by involving in quenching, scavenging and antioxidant reactions.[29-33] The net effect of these photochemical processes depends on the source and characteristics of DOM.[34-37] DOM derived from treated wastewater, usually termed effluent organic matter (EfOM), can produce reactive species,[38-41] thereby enhancing transformation of chemical contaminants and inactivation of pathogens.[18] Wetlands are expected to alter the composition of EfOM via microbial decomposition and photochemical processes,[26, 42] and simultaneously new DOM compounds are produced by biological processes in the wetlands[43, 44]. Thus, the effect of DOM on photochemical processes will be modified by the wetlands.

The goal of this study was to characterize the changes to DOM's light-absorbing and photochemical properties in different types of unit process wetlands, and how these changes

affect the sunlight-mediated inactivation of indicator organisms. In addition, we investigated how well DOM extracts can reproduce the photochemistry of an original water sample, including pathogen indicator photoinactivation. Water was sampled from a pilot-scale unit process wetland (Discovery Bay, CA), including the secondary wastewater that served as influent, and effluents from three distinctive wetland cells that were operated in parallel: an open-water, a cattail and a bulrush cell. A solid phase extraction (SPE) method was employed to isolate wetland DOM and was evaluated for its efficiency to extract naturally occurring photosensitizers. Spectroscopic characterization and irradiation experiments were conducted in whole water samples and the re-dissolved wetland DOM isolates were benchmarked against photosensitizer-free phosphate saline buffer (PBS) and two standard DOMs, Suwannee River fulvic acid (SRFA) and Pony Lake fulvic acid (PLFA). Formation of reactive species ($^3DOM^*$, $^1O_2$ and $^\bullet OH$) by whole waters and their DOM extracts was measured and the light-induced inactivation of a bacterial (*E. faecalis*) and a viral indicator (MS2 coliphage) organism were determined. Finally, the ability of wetland and standard DOMs to associate with the bacterial cells was evaluated. Recent work has investigated both the composition and the photochemistry of DOM in a constructed treatment wetland using comparable spectroscopic and photochemical diagnostic tools as applied in this study[45]. However, to our knowledge this is the first comprehensive investigation of changes in spectroscopic, photochemical and photoinactivating properties of DOM during passage through a wetland system, including a comparison of DOM extracts with the original water samples.

## 2. Materials and methods

### Chemicals, preparation of solutions and analytical methods

Chemicals used are listed in Supporting Information (SI) Text S1. Agents related to *E. faecalis* and MS2 preparation and counting are specified in the *Pathogen indicators* section. Aqueous

solutions including stock solutions, phosphate buffer saline (PBS: 4 mM $NaH_2PO_4$, 16 mM $Na_2HPO_4$, and 10 mM NaCl; pH adjusted to 7.5), and clear water for inactivation experiments with bacteria (20mM $NaH_2PO_4$, 10 mM NaCl; pH adjusted to 7.5) were prepared from ultrapure water (resistivity >18 MΩcm) obtained from a Milli-Q (Millipore) water purification system. Microbial broths were prepared from deionized water. Analytical methods are described in SI Text S2.

**Field site and sampling**

Whole water samples for experiments and DOM isolation were collected at the Discovery Bay unit process wetlands, CA, USA (37°88′77.80″N, 121°58′37.23″E). The pilot scale wetland system consisted of four free-surface flow wetlands (each 400 $m^2$), including an open water cell (approximately 0.2 m depth) without emergent plants and three vegetated cells (approximately 0.5 m depth) [two bulrush (*Scirpus* spp.) cells and a cattail (*Typha* spp.) cell] that were operated in parallel during this study. More information on design and function of the open water cell is provided elsewhere [8, 11, 46-49] During the sampling period each cell received approximately 4.4 $\times 10^{-4}$ $m^3$ $s^{-1}$ of non-disinfected, nitrified secondary (oxidation ditch) wastewater from an adjacent wastewater treatment plant as influent. Baffles installed across the cells reduced short-circuiting, with hydraulic retention times in the range from 1 day for the open water cell to 3 days for the vegetated cells. Water samples were collected in clean 20 L plastic containers or 1 L glass bottles at the inlet of the wetland system and the outlets, transferred to the lab, stabilized by filtration with pre-rinsed 1µm glass fiber filters and either immediately processed or stored in the dark at 4°C until further use. Water parameters are provided in Table S2.

**Extraction of DOM from unit process wetlands**

Wetland DOM was isolated from water samples that were all obtained at the same day using a

145 solid-phase extraction (SPE) method [50], briefly described in the Supporting Information (Text
146 S3).

147 **Spectroscopic characterization of wetland waters, isolated DOM and standard DOM**
148 **solutions**

149 UV-Vis absorption spectra were recorded at the same time as irradiation experiments were
150 conducted (see next section) using sensitizer-free water as a reference. Correction of absorption
151 spectra for nitrate and calculation of specific absorption coefficients $a_{(\lambda)}$ [L $(mgC)^{-1}$ $m^{-1}$] is
152 outlined in SI Text S5. Spectral parameters were selected and calculated based on established
153 approaches for spectroscopic characterization of aquatic DOM.[51-54] $SUVA_{254}$ and $SUVA_{280}$
154 values are defined as $a_{(254)}$ and $a_{(280)}$, respectively. Spectral slopes $S_{(300\text{-}700nm)}$ [$nm^{-1}$] were
155 calculated by non-linear least square fits (Origin 8.5, OriginLab Corporation, Northampton, MA)
156 of absorption data from 300 – 700 nm with a single exponential decay function (reference
157 wavelength 350 nm). $E_2/E_3$ is the ratio of $a_{(250)}$ and $a_{(365)}$.

158 **Irradiation experiments**

159 All irradiation experiments were conducted under defined laboratory conditions using a
160 collimated beam Oriel Solar Simulator (Spectra Physics, serial no. 91194) equipped with a
161 1000W Xe lamp and atmospheric attenuation filter (Spectra Physics, serial no. 81088). Emission
162 wavelengths below 320 nm were reduced using a UVB-filter (Spectra Physics, serial no. 81050).
163 UVB light was blocked to reduce the contribution of direct inactivation. This approach has been
164 used in many prior studies to elucidate indirect exogenous inactivation mechanisms.[13] A few
165 experiments were conducted with full spectrum sunlight (atmospheric filter, serial no. 81017
166 instead of UVB filter) to validate photochemical methods and confirm results with the UVB
167 filter; in these experiments the 280-320 nm wavelength range was fully present, and results

under this condition are indicated. Further details including lamp emission spectra and spectral irradiance data are provided in Text S4 and Figure S1. Irradiation experiments were conducted in 5-cm deep, 100-mL black-painted (Pébéo Porcelaine 150 chalkboard black) uncovered glass beakers containing 50 mL of solution (solution depth, depth = 2.5 cm). Reaction solutions either consisted of whole water samples, DOM-free PBS or PBS that was supplemented with the desired amount of DOM stock-solution (up to 15.0 mg C $L^{-1}$ final concentration). Then either photochemical probe compounds or pathogen indicators (next section) were added. Beakers were immersed in a water bath that was maintained at 20°C and solutions were gently stirred. Dark controls were covered with aluminum foil. Formation of reactive intermediates was measured using furfuryl alcohol (FFA) [50µM] for singlet oxygen $^1O_2$,[55, 56] including a few measurements with the $^1O_2$ quencher histidine [20mM] and 2,4,6-trimethylphenol (TMP) [10 µM] for triplet state DOM ($^3DOM^*$).[27] Details on sampling procedures, and calculations of steady state concentration and quantum yields, including correction for light absorption, are provided in Text S6.

**Inactivation of pathogen indicators**

*Enterococcus (E.) faecalis (ATCC 19433).* The bacterial preparation and sampling procedures are described in Text S7. Experiments with DOM solutions were conducted in duplicate. Controls with clear water were included in every experiment resulting in 20 replicates.

*MS2 coliphage.* Detailed virus [MS2 (ATCC 15597-B1) propagation using *E. coli* $F_{amp}$ host (ATCC 700891)], purification and enumeration methods are described elsewhere [57, 58] and summarized in Text S7. Experiments were conducted in duplicate. Controls with PBS were included in each experiment, resulting in 6 replicates.

*Analysis of inactivation data.* Inactivation rates of *E. faecalis* and MS2 were determined from the

slope of linear regression of $\ln(N/N_o)$ versus either time ($k_{obs}$, $h^{-1}$) or incident photon fluence rate $E_p$ [$k_{photon}$, $m^2$ (mole photons)$^{-1}$]. $k_{photon}$ was used to correct for light intensity incident on the water surface and light screening in the water column. Photon fluence rate $E_p$ refers to the number of photons available for absorption by the indicators and photosensitizers. For *E. faecalis*, the values for $E_p$ were measured over the UV wavelengths (280 – 400 nm), which were shown to be most important for sunlight inactivation of *E. faecalis*.[23, 59, 60] For MS2, the values for $E_p$ were calculated from 280 to 320 nm, because UVB was observed to be most important for MS2 sunlight inactivation.[17, 57] Over the conditions studied, the log reduction in *E. faecalis* ranged from 4.8 to 6.0, and for MS2 ranged from 0.2 to 1.3 after 8 and 10 hours under solar simulated light without UVB, respectively. The inactivation curves resembled those in previous studies.[17, 61] Statistical analysis including linear regressions for pathogen indicator inactivation rates was performed using Origin 8.5.

### Association of *E. faecalis* to DOM

*E. faecalis* association with DOM was determined according to previously published protocols [62-64] and is described in detail in SI Text S9.

## 3. Results and discussion

### Isolation and spectroscopic characterization of wetland DOMs

Wetland DOM was isolated to uncouple matrix effects of whole water photochemical reactivity (DOM effects versus those due to other water constituents) and to obtain DOM for experiments with pathogen indicators. Additionally, the performance of the solid-phase extraction (SPE) method for isolating the light-absorbing, potentially photosensitizing fraction of DOM was evaluated and compared to original whole water samples. Measuring spectroscopic parameters of

whole water samples and corresponding isolates served as a simple means to identify changes that DOM underwent in the wetlands and to assess the SPE method. The DOM fraction that is reversibly adsorbed to SPE cartridges and recoverable during the extraction process is defined as DOM isolate. Solution that passed through SPE cartridges and contained the non-recoverable DOM fraction, which did not adsorb, is defined as SPE discharge.

A typical set of UV-Vis spectra for a whole water sample, the corresponding SPE discharge after different extraction volumes, and the re-dissolved isolates is shown in Figure 1a (cattail wetland effluent). Spectra are provided as the specific absorption coefficient $a_{(\lambda)}$ [L (mg C)$^{-1}$ m$^{-1}$], which is independent of TOC concentration. Lower $a_{(\lambda)}$ values in SPE discharge indicate that SPE was effective in isolating the light-absorbing fraction of the DOM. Higher $a_{(\lambda)}$ values of the DOM isolates compared to the original water samples further show that SPE selectively enriched for the colored fraction of the wetland DOM. The enrichment of light-absorbing DOM during the SPE process is also illustrated by $SUVA_{254}$ (Figure 1c) and the alternative $SUVA_{280}$ (Figure S4a).

Comparison of TOC concentration before and after SPE (Table S3) showed that each cartridge was loaded with approximately 0.8 – 0.9 mM C, which is lower than the maximum recommended load of 2 mM C per cartridge.[50] Assuming negligible loss of more hydrophobic DOM fractions on a mass basis due to irreversible adsorption onto SPE-cartridges, [50] it was calculated that the DOM recovered ranged from 47% - 59% (Table S3) and is within the range of the reported extraction efficiencies of this method for isolating DOM from seawater (43 – 62%)[50] and wastewater (57%).[65] Similar to other DOM extraction methods,[66] full recovery of DOM from water samples with the SPE method was not feasible, including full recovery of potentially photosensitizing moieties. [67]

Specific absorption coefficient spectra for the wetland influent, the effluents of the three different cells and the standard DOMs are shown in Figure 1b. To enable comparison between DOM isolates and whole wetland water data, the latter are corrected for nitrate absorption (For uncorrected spectra refer to Figure S2; the correction is almost indistinguishable). The effluents of both vegetated wetland cells exhibited higher specific absorption than the influent, while the open water effluent had the lowest overall absorption. The optical parameters (Figure 1c – d) of the bulrush and cattail cells show an increase in $SUVA_{254}$ (and $SUVA_{280}$, Figure S4A) and spectral slopes compared to the influent, which indicates that DOM has a slightly higher aromaticity and higher molecular size after passage through the vegetated cells,[53, 54] whereas the opposite trend was observed for the effluent of the open-water cell. $E_2/E_3$ ratios (Figure S4b), which inversely correlate with DOM molecular size[53, 68], indicate increasing molecular size in the vegetated wetlands and a decrease in the open water wetland. Comparison to spectroscopic data of SRFA and PLFA, recognized as fulvic acid end-members of terrestrially/higher-plant derived (allochthonous) and aquatic/microbial derived (autochthonous) aquatic DOM,[69, 70] respectively, suggests that DOM in the vegetated cells became more terrestrial-like, which is in agreement with previous studies [43, 45, 71-73], while DOM became more aquatic-like in the open-water cell. A similar trend was also observed for the isolated wetland DOMs, indicating that alterations of DOM occurring in the wetlands were reflected in the isolates (Figure S3).[74]

The results of these analyses indicate relatively little change in the overall spectroscopic properties of DOM by wetland treatment, perhaps due to the short hydraulic retention time of ~1 to 3 days. However, there was still a notable difference in spectroscopic parameters for vegetated wetland and open wetland water. It should be noted that the presented data are for fall/early winter conditions and that the observed spectroscopic trends may vary over season. For example,

the open water wetland effluent DOM could display even more distinct aquatic/microbial properties during spring and summer conditions, when photosynthetic activity is higher. Similarly, higher temperatures during summer causing faster decay of plant litter may increase release of plant derived DOM from the vegetated cells, thereby increasing its terrestrial/higher-plant derived character.

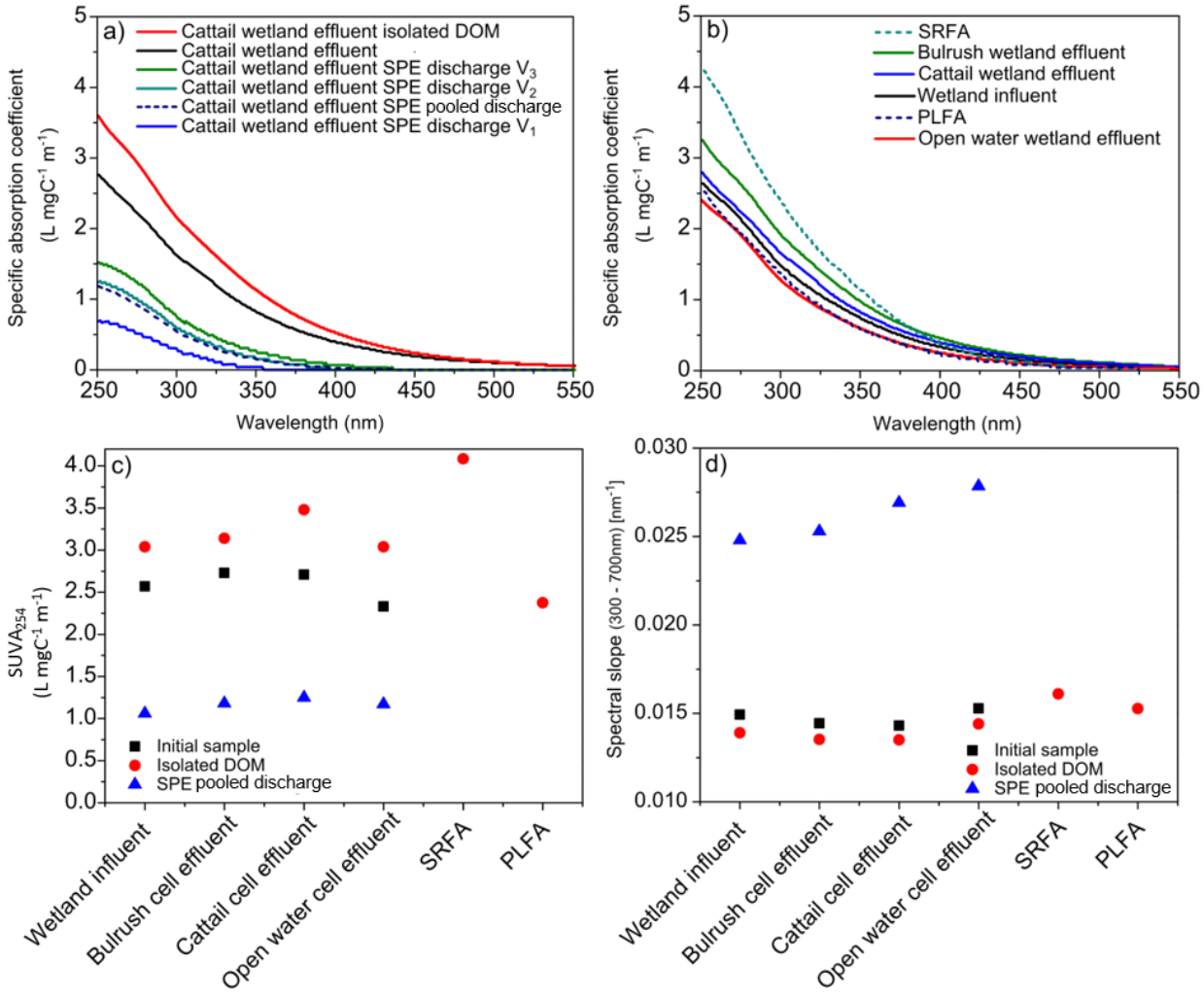



**Figure 1.** Spectral characteristics of whole water samples, DOM isolates and standard DOMs: (a) Exemplary set of spectra of cattail wetland effluent during SPE [$V_1$,$V_2$ and $V_3$ are spectra of the discharge after 0.1 L, 0.5 L and 2 L water passed through the SPE-column and the pooled

discharge; (b) Specific absorption coefficient spectra $a_{(\lambda)}$ [L (mg C)$^{-1}$ m$^{-1}$] of whole water samples; (c) $SUVA_{254}$ and (d) Spectral slopes $S_{(300\text{-}700nm)}$. Data shown are corrected for nitrate absorption, if applicable.

**Photochemical reactive species formation in wetland waters and their DOM isolates**

To examine the importance of non-recoverable chromophores during SPE and to better understand the alteration of the DOM photochemistry during wetland passage, comparative measurements of photochemical formation of $^1O_2$, $^3DOM^*$ and $^\bullet OH$ in the whole water samples, the (pooled) SPE discharges, and the DOM isolates were conducted. The related depletion kinetics data of the $^1O_2$ probe FFA and the $^3DOM^*$ probe compound TMP, including quenching measurements, for the wetland influent and the effluents of the different wetland cells are provided in the SI (Figures S5-S9). The irradiated whole water samples and the isolates show comparable FFA depletion kinetics at similar TOC concentration and pH (see also Figures 2a, S11 and S12), which confirms previous findings that under standardized conditions SPE extracts highly represent both the optical and photochemical properties of their origin waters.[74] The FFA depletion was efficiently suppressed by the $^1O_2$ quencher histidine. Also, there was no FFA depletion observed in the SPE discharges under both UVB-blocked and full spectrum simulated sunlight (Figure S9), indicating that the SPE method was efficient in the extraction of $^1O_2$ sensitizing moieties.

TMP depletion in a given DOM isolate solution was always faster than in the corresponding water sample and there was still a considerable decline of TMP in the irradiated SPE discharges (Figures S5 – S9), possibly indicating triplet precursors were still present in the SPE discharge. Intersystem crossing quantum yields ($\Phi_{ISC}$) ranged from 2.5% to 3.3% (Table S4) for SPE

discharge samples, which is 2.8 – 6.2 times higher than $\Phi_{ISC}$ for both whole water samples and isolates (Figure 2b), similar to quantum yield coefficients $f_{TMP}$ (Figure S13, Table S4). This could suggest the presence of a pool of SPE non-recoverable triplet precursors in the water samples whose excited states are highly reactive with TMP but do not lead to the production of $^1O_2$. However, this would disagree with recent findings that most $^3DOM^*$ readily forms $^1O_2$.[75, 76] A possible explanation is that quenching by selectively enriched DOM constituents in the SPE discharge prevents transfer of triplet states to $^1O_2$. For example, higher phenolic content in DOM may lead to lower $^1O_2$ quantum yields due to quenching of $^3DOM^*$.[75] The phenolic probe TMP competes for the same pool of reactants. In fact, tests with the triplet state quencher sorbic acid showed a lower relative decrease in TMP depletion in the SPE discharge, along with a higher relative decrease for the isolates in comparison with the original water samples (SI Figure S10), indicating selective enrichment of triplet quenchers in the discharge. However, under air-saturated conditions molecular oxygen should be the major quencher of $^3DOM^*$ to form $^1O_2$. Alternatively, SPE discharge may form superoxide more efficiently and thereby DOM radicals which could result in a different TMP transformation pathway.[77] A deeper investigation was outside the scope of this study but the results indicate that SPE separation of DOM can be useful in future studies to further elucidate the nature of the different triplet state precursors of DOM and their photochemical reaction pathways.[74-76, 78]

Figure 2a shows light-absorption corrected data of singlet oxygen steady-state concentration $[^1O_2]_{ss}$ and TMP-depletion rate constants $k_{TMP}$ for different water samples of the cattail wetland effluent, including results for different concentrations of the isolated DOM (similar datasets for influent, other effluents and standard DOMs see Figures S11 and S12.) $[^1O_2]_{ss}$ increased linearly with increasing DOM concentration (Table S5), whereas $k_{TMP}$, despite correction for light-

313 absorption, tended to level off slightly at higher DOM concentrations. Inhibition of TMP
314 oxidation at high DOM concentration was observed before and has been ascribed to self-
315 quenching of DOM or antioxidant effects.[74, 76, 79, 80] Although, antioxidant effects are not
316 obviously affecting TMP oxidation at low DOM concentrations of 2.3 mg C $L^{-1}$ and 3.5 mg C $L^{-1}$,
317 respectively. [37, 80]. Triplet state quenching by DOM is expected to be insignificant below DOM
318 concentrations of 22 – 72 mg C $L^{-1}$.[32]
319 Singlet oxygen and ISC quantum yield trends align. Values for influent and open water isolates
320 and whole water differ only slightly, in agreement with Maizel & Remucal[74], while quantum
321 yields for the vegetated wetland isolates were higher than for the corresponding water samples.
322 Also, quantum yields were higher for influent and open water effluent compared to the vegetated
323 cells. Others have observed an increase in PPRI formation of DOM after passage through
324 wetland systems and ascribed this to the contribution of terrestrial-type DOM released into the
325 wetland by decaying plant material[45, 81]. Here the decrease in quantum yields for the vegetated
326 wetland cells can be at least partly attributed to the release of light-absorbing DOM not serving
327 as reactive species precursor. Additionally, the plant derived DOM could act as triplet state
328 quencher and thereby decrease quantum yields. For the open water wetland DOM the loss of
329 specific $^1O_2$ quenchers by photobleaching could explain the increase in $^1O_2$ quantum yield [26].
330 Photobleaching is also suggested by the decreasing $SUVA_{280}$ and increasing $E_2/E_3$ ratios [26, 53, 82]
331 (Figure S4), indicating decreased aromaticity and molecular weight [26, 51], while these trends were
332 opposite for the vegetated cells. Our results agree with previous studies showing correlation
333 between increasing $E_2/E_3$ ratios and both $^1O_2$-quantum yields and $f_{TMP}$, respectively. [38, 68, 83]
334 Increasing $E_2/E_3$ indicate loss of light-absorbing charge transfer complexes that reduce the
335 efficiency of $^3DOM^*$ formation.[26, 84] Standard DOM quantum yields and $f_{TMP}$ (Figure S13)

compare to literature values despite lower $^1O_2$-quantum yields and $f_{TMP}$ (both integration range 280 nm – 700 nm, Text S6 SI) than previously found,[26, 38, 75, 85] Tests with scavengers and probe compounds showed that •OH played no role during singlet oxygen formation in the irradiation system (Text S10, Figures S14-S15).

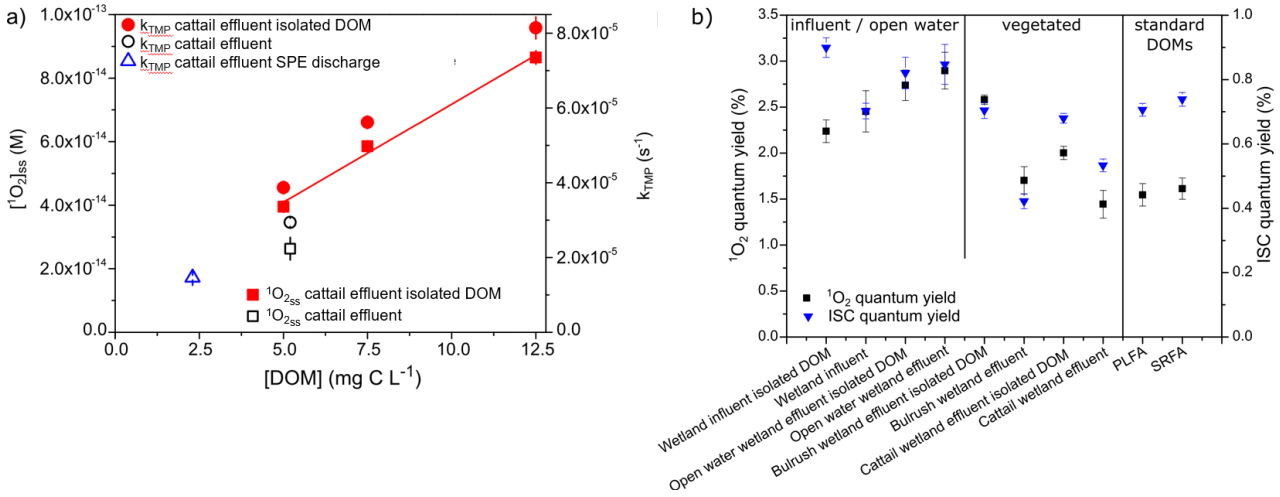



**Figure 2.** (a) Steady state singlet oxygen concentrations ($^1O_{2ss}$) and TMP depletion rate constants ($k_{TMP}$) vs DOM concentration for cattail wetland effluent samples. (b) Singlet oxygen ($\Phi_{1O2}$) and intersystem crossing ($\Phi_{ISC}$) quantum yields for DOM isolates and standard DOMs (determined at 5 mg C L$^{-1}$). Note, $\Phi_{1O2}$ and $\Phi_{ISC}$ may not be directly comparable due to use of different probe molecules and averaged rate constants for $\Phi_{ISC}$ calculation (for details see Text S6).

## Inactivation of pathogen indicators for wetland and standard DOM isolates

Consistent with previous research, photoinactivation rates of *E. faecalis* were higher than MS2 for all wetland DOM isolates and standard DOMs[9, 10] (Figure 3a and 3c). Figure 3b and 3d provide the corresponding relationship between the measured singlet oxygen concentrations and the inactivation rates. Results uncorrected for light screening are shown in Figure S16. Inactivation rates for DOM solutions are shown in comparison to the rates in sensitizer-free

352 solutions (PBS without addition of DOM) to isolate the effects of DOM and other water
353 constituents. MS2 results are discussed first followed by *E. faecalis*.

354 Note that for MS2, an initial increase of the virus count over time was observed in dark controls,
355 sensitizer-free solutions and a few experiments with the lowest used DOM concentration of 5 mg
356 C $L^{-1}$. This phenomenon has been noticed previously [86, 87] and is assumed to occur due to break-
357 up of virus aggregates. With the plate-based counting method employed in this study virus
358 aggregates cannot be distinguished from single viruses as both appear as single plaque leading to
359 an underestimation of total plaque forming units (PFU) in a given sample, while the actual
360 number of viruses could be decreasing due to photoinactivation. To avoid introducing further
361 biases, all data points were used for calculation of inactivation rate constants, resulting in
362 negative rate constants for a few samples. Despite these methodological issues we believe the
363 results for MS2 are internally consistent, allowing us to interpret the overall trends.

364 For MS2, the lowest inactivation rates were observed in sensitizer-free PBS. For all solutions
365 containing DOM isolates, inactivation rates of MS2 (including rates uncorrected for light
366 screening) increased with increasing DOM concentration, indicating that the photosensitizing
367 effects of DOM were larger than its role in attenuating light. Wetland influent DOM was a more
368 effective photosensitizer for MS2 inactivation, while inactivation rates for the three wetland
369 effluent isolates and the standard DOMs were within a similar range. The slopes derived from
370 linear regression of MS2 inactivation rates and DOM concentration were between 6.73 – 19.5 $m^2$
371 $(\text{mole photons})^{-1}/(\text{mg C L}^{-1})$ and for linear regression with singlet oxygen concentration were
372 between 16.0 – 28.8 $m^2$ $(\text{mole photons})^{-1}/(10^{-14}\ \text{M}\ {}^1O_2)$. The two slopes (vs DOM concentration
373 and vs singlet oxygen) are similar (Table S6) for open water wetland effluent, bulrush wetland
374 effluent and SRFA. In contrast, the singlet oxygen calculated slopes were higher for wetland

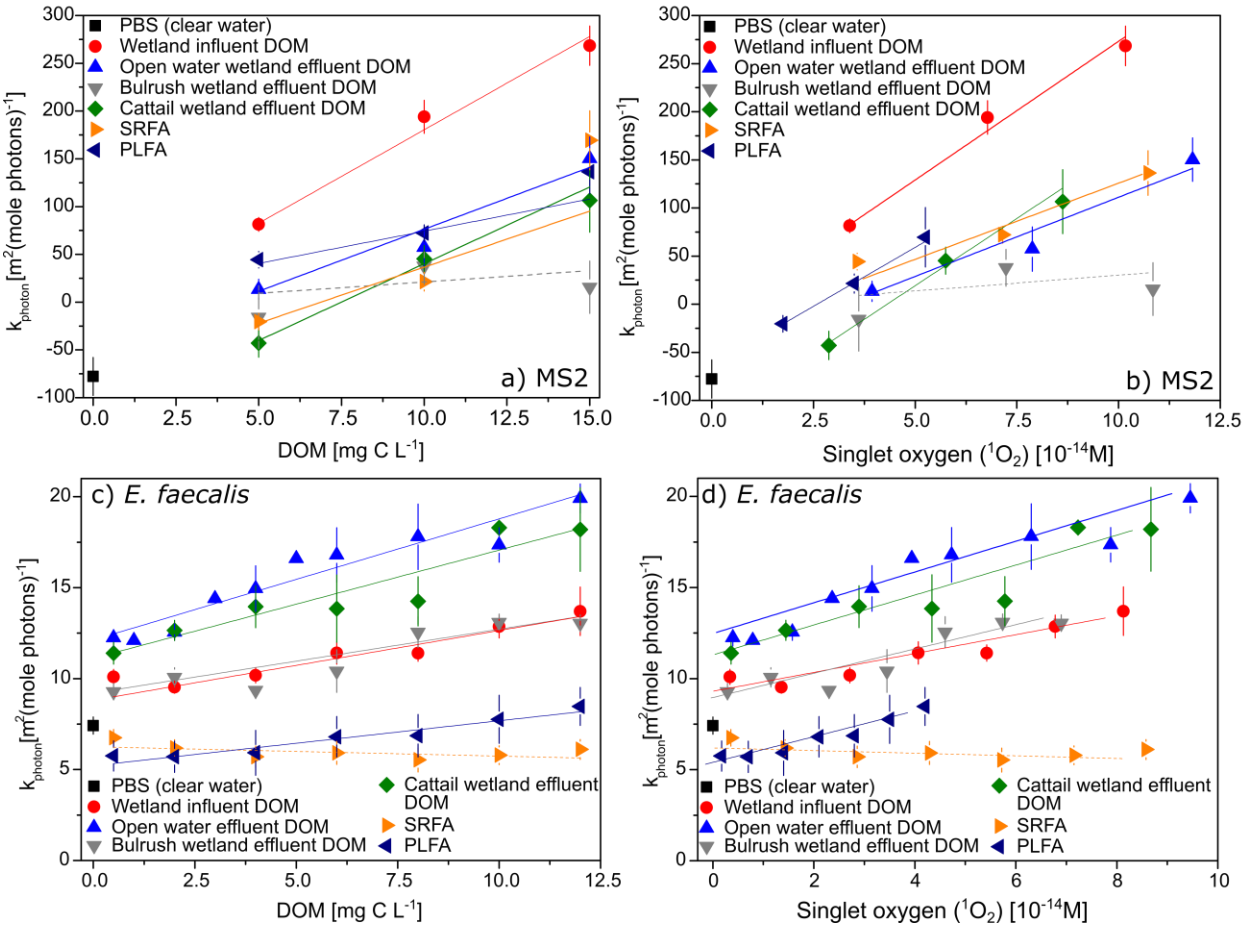
influent, cattail effluent and PLFA. Predicted intercepts (assuming absence of photosensitizers) were between -120.5 ± 16.0 $m^2$ (mole photons)$^{-1}$ and -15.0 ± 13.5 $m^2$(mole photons)$^{-1}$, while actual values for PBS were -77.8 ± 19.9 $m^2$(mole photons)$^{-1}$. Consistent with previous reports, the high correlation coefficients for singlet oxygen provide further evidence that steady-state bulk water singlet oxygen concentration can be used to predict the MS2 photoinactivation rate, although the correlation is unique to each water or sensitizer type[88-91]. However, in absence of singlet oxygen measurements DOM concentration may also serve as a reasonable indicator to predict photoinactivation of MS2. For the tested range of 5 – 15 mg C $L^{-1}$ simple linear fits seemed to adequately describe MS2 photoinactivation with DOM. However, for wider DOM concentrations ranges, including data at low DOM concentration, Langmuir-type adsorption curves describe MS2 photoinactivation more accurately because such fits account for saturation of MS2 with photosensitizers[92]. Bulrush wetland effluent data were treated as outlier and excluded from the data interpretation. Linear regression analysis using TMP transformation data did not return reasonable results due to the non-linear effects of TMP depletion at higher DOM concentration, that are discussed in the previous section. The contribution of hydroxyl radicals was assumed to be unimportant for pathogen indicator inactivation under the experimental conditions employed (see previous section).

For *E. faecalis*, inactivation corrected for light screening increased with increasing DOM (Figure 3c) and singlet oxygen concentration (Figure 3d), respectively, in the presence of wetland isolates and PLFA. Uncorrected data show little increase with increasing DOM concentration indicating that light-screening almost entirely compensated for the increase in photoinactivation (Figure S16c/d). For SRFA, light screening was the dominant effect and even corrected data show a slightly decreasing trend at higher DOM concentrations (Figure 3d). SRFA is not further

considered in the discussion of the regression analysis. Linear regression data quality (lines shown in Figure 3c/d) was almost the same for inactivation rates vs DOM and singlet oxygen concentration, respectively.

Results for *E. faecalis* at low DOM concentrations of 0.5 mg C $L^{-1}$ exhibited inactivation rates across different datasets that appear markedly different from inactivation data in PBS. However, the overall range of inactivation rates at this DOM concentration (5.75 – 12.25 $m^2$ (mole photons)$^{-1}$) was comparable to the range observed for PBS (3.5 – 11.5 $m^2$ (mole photons)$^{-1}$). For PBS small standard deviation stems from a high number of repetitions (N=20). Given this observation we suggest that the offset of DOM data compared to PBS data is due to experimental variation rather than sudden changes in phototoxicity upon addition of small amounts of DOM. Internally datasets are consistent, while the PBS value should serve as a benchmark value for inactivation in sensitizer free water during the following discussion. Inactivation of *E. faecalis* under dark condition in clear water and with added DOM was insignificant ($k_{dark}$ = 0.06±0.08 $h^{-1}$).

In summary, the photoinactivation of the two pathogen indicator organisms shows a concentration dependence with the measured singlet oxygen concentration (and DOM concentration), which is linear in the tested range of up to 15 mg C $L^{-1}$. Slopes of regression analysis for singlet oxygen concentration as a measure for photoinactivation efficiency differ by 80% for MS2 and 50% for *E. faecalis*, respectively (Tables S6/S7), which is small when considering that logarithmic scale inactivation over several orders of magnitude for pathogen organisms is desired. This also means that there is overall little difference of photoinactivation action between the different wetland isolates and the standard isolates, except SRFA for *E. faecalis*.




421

422 **Figure 3.** Inactivation rates of pathogen indicators in pure water and for constructed wetland
423 isolates and standard DOMs with UVB-blocked simulated sunlight corrected for light screening
424 vs DOM concentration (left hand side figures) and measured singlet oxygen concentration (right-
425 hand side figures). (a)/(b) MS2 (c)/(d) *E. faecalis*. Y-axis error bars indicate ± standard error of
426 replicate experiments. Some error bars are smaller than data points. X-axis errors for singlet
427 oxygen concentration (<10%) not shown. $k_{photon}$ calculated using photon fluence of inactivation
428 action spectra of 280 – 320 nm for MS2 and 280 – 400 nm for *E. faecalis*, respectively.
429 Numerical results of the linear regression analysis are provided in Tables S6 and S7.

430

**Pathogen indicator inactivation in original water samples compared to DOM isolates, SPE discharge and reconstituted solutions.**

The effect of DOM separation by SPE (into an isolated fraction and a non-recoverable fraction) on pathogen indicator inactivation was assessed by comparing inactivation rates of whole water (original samples), SPE discharges and the DOM isolates (Figures 4 and S16-S17 [uncorrected data]). In addition, experiments with MS2 were conducted with reconstituted wetland water (Figure 4a). Reconstituted wetland water was prepared by adding an appropriate amount of isolated DOM to the corresponding SPE discharge to reestablish the initial DOM concentration and by readjusting pH.

Both MS2 and *E. faecalis* inactivation rates in SPE discharge were found to be small but slightly higher than in sensitizer free PBS. These results are in good agreement with results presented in the previous section showing the absence of $^1O_2$ in irradiated SPE discharge samples and further reinforce the importance of $^1O_2$ (or other species that scale with $^1O_2$) as major contributor in the exogenous inactivation of pathogens[88, 93-95]. Inactivation in SPE discharge was still higher than in PBS, indicating some importance of the remaining water constituents on photoinactivation. The highest inactivation rates for MS2 were observed in whole wetland waters, followed by DOM isolates, while rates in reconstituted wetland water were much lower and similar to SPE discharge. This discrepancy was surprising, as inactivation rates in reconstituted wetland water were expected to be closer to the original samples, revealing that reconstituting wetland water did not reestablish inactivation conditions of whole water samples. A possible explanation is that the SPE extraction procedure irreversibly disturbed the integrity of the initial DOM molecule assemblies by separating DOM oligomers that were loosely connected via non-bonded van der Waals interactions, hydrogen bonds or electrostatic interactions.[96] While this did not seem to

affect the DOM moieties responsible for photochemical singlet oxygen production, as discussed in the previous section, the initial DOM assemblies seem to be important facilitators of MS2 inactivation. We hypothesize that the initial DOM trans-molecular structure allows a closer proximity of MS2 via adsorptive interactions [97] to DOM regions with locally higher singlet oxygen concentration. [98, 99] Additionally, the photoreactive constituents left in the SPE discharge could be involved in MS2 inactivation via direct reaction of their excited triplet states with MS2. In this study higher MS2 inactivation in solutions of wastewater derived DOM may be due to higher association between this type of organic matter and viral particles compared to other DOM isolates. It was previously shown that close proximity between DOM and MS2 particles results in exposure to higher concentration of $^1O_2$ and an increase in MS2 inactivation.[92]

Reestablishing the pH with NaOH after acidification for SPE extraction with HCl increased sodium chloride concentrations in reconstituted water, resulting in chloride concentration of approximately 1000 – 1500 mg $L^{-1}$ compared to 362 – 392 mg $L^{-1}$ in the initial wetland samples (Table S2). Although halide-ion concentration has been shown to impact the photochemistry of natural organic matter[100], including quenching of $^1O_2$, we believe the increase in chloride in our experiments is of minor importance for photoreactivity and inactivation of MS2. On the other hand, other water constituents such as divalent cations may play a role in the observed lower MS2 inactivation in experiments with DOM isolates. For example, concentrations of divalent cations ($Mg^{2+}$ and $Ca^{2+}$) in wetland isolated DOM solutions were negligible and approximately 2,000 times lower than in the whole wetland waters (Table S2). Such high divalent cation concentrations in wetland waters might increase virus-photosensitizer association, and sunlight inactivation, through cation bridging.[92] Previous studies reported that the MS2 apparent second-order rate constant with $^1O_2$ ($k_2$) in the open-water wetland effluent was found to be significantly

higher than $k_2$ observed in other environmental waters.[9, 17, 20] One of the explanations given for such high $k_2$ value in the open-water wetland effluent was the relatively high divalent cation concentration of the wetland water.[9]

In contrast to MS2, the inactivation rates for *E. faecalis* in original water samples were close to the rates with DOM isolates. This difference suggests that macromolecular structures of DOM may not be relevant for *E. faecalis* inactivation, which is possibly due to the much larger size of the bacterial pathogen indicator. The size of MS2 is 27 nm[101], while the size of *Enterococcus spp.* is between 600 nm and 2500 nm[102]. In summary the results of this section suggest that exogenous photoinactivation rate estimates for *E. faecalis* based on bulk singlet oxygen concentration data or experiments with DOM isolates could be transferable to other natural water bodies and treatment wetlands. To quantify MS2 photoinactivation it is recommended to conduct experiments in the original water samples.

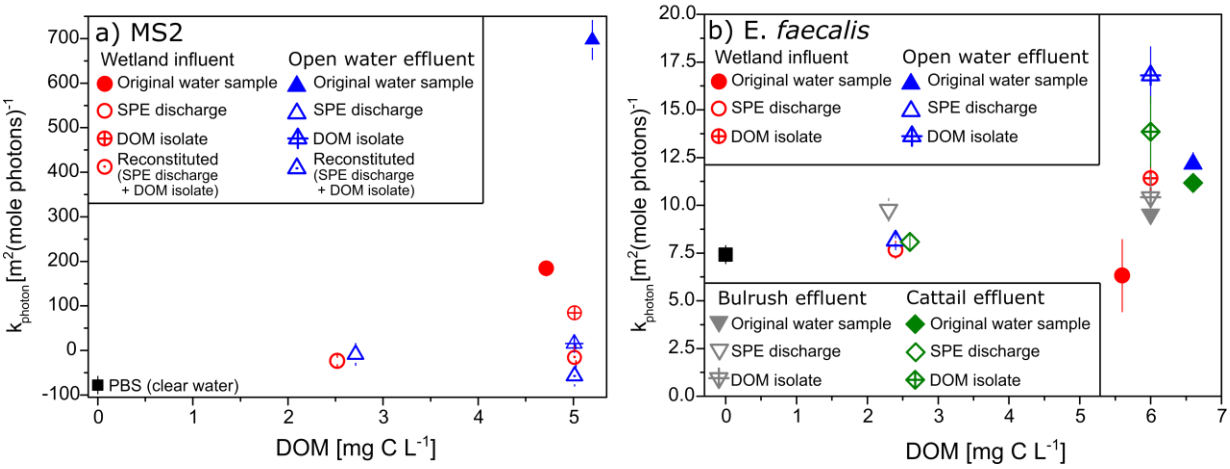



**Figure 4.** (a) MS2 photoinactivation rates in original water samples, SPE discharge, DOM isolates and reconstituted solutions for wetland influent and open water effluent. (b) *E. faecalis* photoinactivation rates for original water samples, SPE discharge and DOM isolates. Data corrected for light-screening. Error bars indicate ± standard error of replicate experiments. $k_{photon}$

calculated using photon fluence of inactivation action spectra of 280 – 320 nm for MS2 and 280 – 400 nm for *E. faecalis*, respectively.

**Association of *E. faecalis* to DOM isolates.**

Previous research has shown that association of DOM with MS2 enhances exogenous photoinactivation because photo-produced reactive intermediates (PPRI) are in closer proximity to targets of damage[92, 103]. To explore whether a similar phenomenon occurs with *E. faecalis*, the association between DOM isolates and *E. faecalis* cells was measured and plotted against inactivation rates. Between 49 – 72% of the wetland DOMs associated with *E. faecalis*, compared to 15 – 19% for the standard DOMs (x-axis, Figure 5 and S18). There was also a positive linear correlation between the corrected inactivation rate and DOM concentration for all wetland isolates (Figure 3c). The slope of this line ($k_{photon}$/mg C $L^{-1}$ of DOM) is plotted as the y axis in Figure 5. The results presented in Figure 5 suggests a rough correlation between the amount of DOM associated with bacterial cells and inactivation rates, with correlation coefficients of $R^2 = 0.78$ when including SRFA data and $R^2 = 0.61$ when excluding SRFA. This correlation indicates that a higher degree of association of DOM with *E. faecalis* cells appears to increase photoinactivation.

The ability of DOM samples to associate with the bacterial cells may be affected by several factors, such as the concentration of cations in the DOM samples[92], or the ability of *E. faecalis* cells to uptake DOM actively via protein transporters.[104] Concentration of bivalent cations in DOM isolates and PLFA is similar and in fact highest for SRFA (Table S2) ruling out that this a factor that caused enhanced association with *E. faecalis* cells, including higher inactivation compared to SRFA. Association of photosensitizers with target bacteria has been shown to increase the efficiency of photosensitizers. For example, synthetic cationic porphyrins are more

efficient than anionic porphyrins in the photoinactivation of both gram-positive and gram-negative bacteria[105]. A similar relationship may apply to natural and waste water derived photosensitizers occurring in surface waters. DOM with higher molecular weight was more hydrophobic and associated more with gram positive bacterial cells.[62, 64] While hydrophobic interactions are likely involved in the association of DOM isolates with *E. faecalis* additional characterization of the DOM isolates is needed and this topic is suggested as an area for future research.

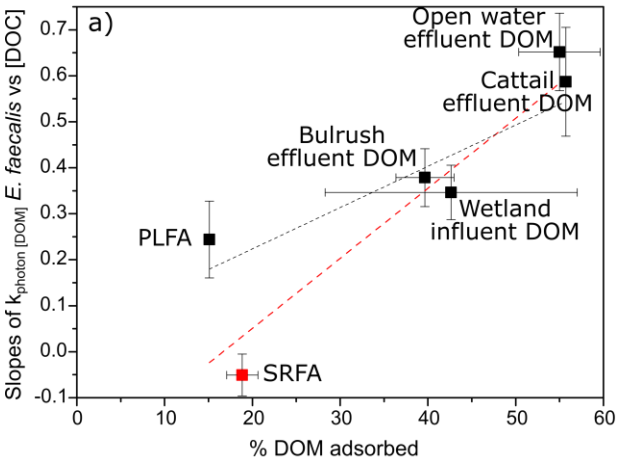



**Figure 5.** Slopes of inactivation rates of *E. faecalis* vs amount of DOM associated with *E. faecalis* ([TOC] = 25 mgC $L^{-1}$; ~$10^{10}$ CFU $mL^{-1}$). Dashed lines indicate linear regression between $k_{photon}$ for all isolates and the percentage of associated DOM. The black line is excluding results for SRFA. X-error bars indicate ± one standard error of replicate measurements. Y-error bars indicate standard error of regression results for $k_{photon}$. Some error bars are smaller than symbols

**Implications for photoinactivation research and wetland design**

SPE was found suitable for isolating DOM from wastewater treatment wetlands. Relatively minor spectroscopic and photochemical alterations of the DOM caused by treatment were reflected in the isolates. This ability to isolate DOM makes it possible to conduct extensive studies on the temporal and spatial changes in the photochemistry of organic matter within and across aquatic systems. On the other hand, because a large difference was observed between the inactivation rate of MS2 in secondary wastewater (wetland influent) and its DOM isolate, care should be taken in extrapolating MS2 photoinactivation results from simple matrices to whole environmental waters. Interestingly, we did not observe net formation of effective natural photosensitizers in the vegetated wetlands, while the partial transformation of the DOM during the wetland passage led to a higher light absorption. To achieve high sunlight-induced disinfection rates of pathogens in polishing wetlands, there does not appear to be an advantage to placing vegetated wetlands prior to an open water wetland. In fact, it may be beneficial to place the open water wetland upstream of vegetated wetlands.

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## Notes

The authors declare no competing financial interests.

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## Supporting Information

10 text files on analytical methods, solid phase extraction (SPE) details, spectral analysis of wetland samples and standard DOM solutions, reactive species measurements, calculation of quantum yields and correction factors, *E. faecalis* and MS2 preparation and counting. 7 tables showing HPLC analytical parameters, water pH and ions analytical results, SPE TOC mass balances, quantum yield coefficients for SPE discharge, steady state singlet oxygen concentrations for measurements with wetland water and linear regression analysis of pathogen indicator inactivation. 17 figures showing lamps emission spectra, UV-Vis absorption spectra of water samples including additional SUVA data, depletion or formation kinetics of photochemical probe compounds, steady-state singlet oxygen concentrations and TMP depletion rate constants, singlet oxygen quantum yields in presence of methanol, pathogen indicator inactivation results (uncorrected for light absorption) and DOM association with *E. faecalis*.

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